

Smaller damselflies have better flight performance at lower body temperature: implications for microhabitat segregation of sympatric *Mnais* damselflies

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In many cases where two closely related species coexist, ecological interaction or reproductive interference drive species to diversify in their body size and/or other signal traits, often concurrently with microhabitat segregation. However, it is usually unclear how character diversification is associated with microhabitat segregation. We performed laboratory experiments using males of two damselfly species (*Mnais costalis* and *Mnais pruinosa*) collected from a syntopic site in Shiga Prefecture, Japan. We analyzed the effects of body temperature and body size on three indices of flight performance: wing-beat frequency and flight speed as measures of thrust production, and minimum body temperature for flight (MBTF). The results showed that the MBTF was correlated with body size: the smaller species (*M. pruinosa*) flew better than the larger species (*M. costalis*) in a cool environment. The initial flight speed was positively correlated with body temperature, but negatively correlated with body size. The wing-beat frequency was also positively correlated with body temperature, but negatively correlated with body size. The combined effects of body size and body temperature on wing-beat frequency meant that overall, there was no significant difference in initial flight speed. We suggest that the effect that body size and temperature have on flight performance explains the previously documented microhabitat segregation occurring between these two species, with the larger *M. costalis* preferring sunny environments and *M. pruinosa* preferring shady environments.

Keywords: Odonata; *Mnais costalis*; *Mnais pruinosa*; microhabitat preference; thermal environment; body size; flight performance

Introduction

Microhabitat partitioning in space or time is a widespread mechanism of coexistence (Cody & Brown, 1969; Konuma & Chiba, 2007; Kuno, 1992; McLain & Pratt, 1999; McLain & Shure, 1987; Okuyama, Samejima, & Tsubaki, 2013; Singer, 1990). However, it is usually difficult to show whether resource competition or interspecific sexual interaction is the direct cause of microhabitat partitioning. This is because species-specific difference in physiological responses may proximately influence microhabitat preference and microhabitat segregation (Huey, 1991). Particularly in ectothermic animals, the thermal environment is often one of most important factors in choice of microhabitat. This is probably because environmentally dependent body temperature influences physiological and behavioral activities (Heinrich, 1981, 1993), and individual fitness (Huey, 1991; Huey & Berrigan, 2001; Tsubaki, Samejima, & Siva-Jothy, 2010;

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Willmer, 1991). We have previously shown that in the field *M. costalis* males have higher body temperatures, reflecting their microhabitat preference for sunny sites, while *M. pruinosa* males have lower body temperatures, reflecting their preference for shady environments (Okuyama et al., 2013).

In our previous paper (Okuyama et al., 2013), we demonstrated microhabitat segregation in sympatric *Mnais* damselflies (Odonata: Calopterygidae). Males of both species hold territories within the same stretch of the river in most of Kinki region in central Japan. However, *M. costalis* were more abundant on the lower stream, while *M. pruinosa* were more abundant on the upper stream (Nomakuchi, 1992; Okuyama et al., 2013; Suzuki & Tamaishi, 1982). Measurement of canopy openness using hemispherical photographs with a fisheye converter revealed that *M. costalis* preferred sunny microhabitats while *M. pruinosa* preferred shady microhabitats (Okuyama et al., 2013). Analyses of the overlap in distributions using Morisita's R_δ index (Morisita, 1959) showed that there was no significant effect of male density of either species (Okuyama et al., 2013). These observations suggest that the two species are segregated in microhabitat due to different thermal preferences, rather than as a result of agonistic interspecific interactions (Okuyama et al., 2013).

Mnais costalis and *M. pruinosa* are very closely related species (Dumont, Vanfleteren, Jonckheere, & Weekers, 2005; Hayashi, Dobashi, & Futahashi, 2004) and are endemic to Japan, with *M. costalis* allopatrically distributed in the north (e.g. the Hokkaido, Tohoku and northern Kanto regions), and *M. pruinosa* allopatrically distributed in the south (e.g. Pacific sides of the Kanto, Chubu, Kinki and Sikoku regions). The two species coexist in the central region from Chubu to Kyusyu. Adults of both species emerge from late April to July, and inhabit forest streams of low mountains. In allopatric populations, males of each species exhibit genetic polymorphisms in wing color linked to alternative mating strategies (Tsubaki, 2003): larger orange-winged males and smaller clear-winged males. Orange-winged males show territorial behaviors, defending dead semi-submerged logs or emergent plants into which females oviposit, and copulate with females on territories. Clear-winged males usually perch nearby orange-winged males' territorial sites, trying to intercept approaching females for copulation and oviposition (for *M. costalis*; Hayashi et al., 2004; Tsubaki, Hooper, & Siva-Jothy, 1997; for *M. pruinosa*; Nomakuchi, 1992; Nomakuchi, Higashi, & Maeda, 1988).

In contrast, sympatric populations exhibit regional differences in the pattern of male wing color polymorphism (Hayashi et al., 2004; Suzuki, 1984). In most of the region of Chubu and Kinki including our study site, both species show sympatric distribution and their male polymorphism disappears: *M. costalis* males are monomorphically orange-winged, while *M. pruinosa* males are monomorphically clear-winged. *M. costalis* are mainly found in open, sunny lower streams whereas *M. pruinosa* mainly inhabit shadier, upper streams (Okuyama et al., 2013).

In this study, we conducted laboratory experiments to test the hypothesis that the microhabitat preferences of the two *Mnais* species correspond with differences in the effect that body temperature and body size have on flight performance, measured as minimum body temperature for flight (MBTF), and initial flight speed and wing beat frequency (as measures of thrust production).

Methods

Minimum body temperature for flight (MBTF)

Males of *M. costalis* ($n = 7$ in 2007; $n = 5$ in 2010) and *M. pruinosa* ($n = 10$ in 2007; $n = 4$ in 2010) were collected in May from Shiga prefecture where the two species occur in sympatry. Left hind wing length and abdomen length were used as indicators of body size.

Prior to flight experiments, the damselflies were cooled down in the refrigerator to about 10°C. Individuals were then removed from the refrigerator and flight trials were conducted to determine the minimum body temperature for flight (MBTF). In each flight trial, we measured the thoracic surface temperature using an infrared thermographic camera (Chino CPA-1000, CHINO, Japan), as a measure of body temperature. We then dropped a damselfly from a height of 1.5 m, allowing it to fly freely in the laboratory room, and observed if it could fly at least horizontally or not. These flight trials were repeated until the individual's body temperature rose to a marginal level for horizontal flight with the last measurement for each individual being recorded as the MBFT. All trials were conducted in the laboratory under the air temperature condition at 25°C.

We performed *t*-tests to compare the average body size (left hind wing length and abdomen length) and the mean MBFT.

Initial thrust production

Males of *M. costalis* ($n = 6$ in 2010; $n = 2$ in 2011) and *M. pruinosa* ($n = 17$ in 2010; $n = 1$ in 2011) were collected and their body sizes were measured as above.

The thrust production experiment was performed in the same laboratory as the MBTF experiments at a constant air temperature of 25°C. In order to measure initial thrust production we prepared a white board (1 m wide and 0.7 m high) with scale marks at 0.1 m intervals in the middle of the room, placing the right hand side of the board toward the direction of the window. Damselflies released at the left-hand (dark) side of the board typically flew straight toward the window. We recorded their flights passing transversely across the board with a high speed video camera (Casio EX-F1, CASIO, Japan, 150-frames s⁻¹), which allowed us to quantify flight speed and number of wing-beats during flight. The flight speed was calculated as the reciprocal of the required time for a damselfly to pass the board. Prior to each flight trial we manipulated the body temperature of damselflies by either cooling them down in the refrigerator or warming them up using an incandescent light bulb. We measured the thoracic surface temperature of each individual before each flight trial using an infrared thermographic camera (Chino CPA-1000, CHINO, Japan). Some individuals were recorded multiple times, but only the best recording (when it flew in a straight line) was used for the analysis.

We performed analysis of covariance (ANCOVA) to test for the effects of body size, body temperature and species identity on the flight speed and the wing beat frequency. For body size, we used the length of left hind wings, because there was the correlation between abdominal length and left hind wing length (see Results). All analyses were performed using R 2.9.2 (<http://www.r-project.org/>).

Results

Male body size

Abdomen length was plotted against left hind wing length of males in *Mnais* damselflies collected from our study site in Figure 1. The average abdomen length (mean = 44.75, SE = 0.38, $n = 26$) and the left hind wing length (mean = 37.65, SE = 0.40, $n = 26$) were significantly larger in *M. costalis* than those (abdomen length: mean = 41.29, SE = 0.26, $n = 35$, left hind wing length: mean = 33.50, SE = 0.25, $n = 35$) of *M. pruinosa* (t value = 7.73, $p < 0.0001$ for abdomen length, and t value = 9.20, $p < 0.0001$ for left hind wing length).

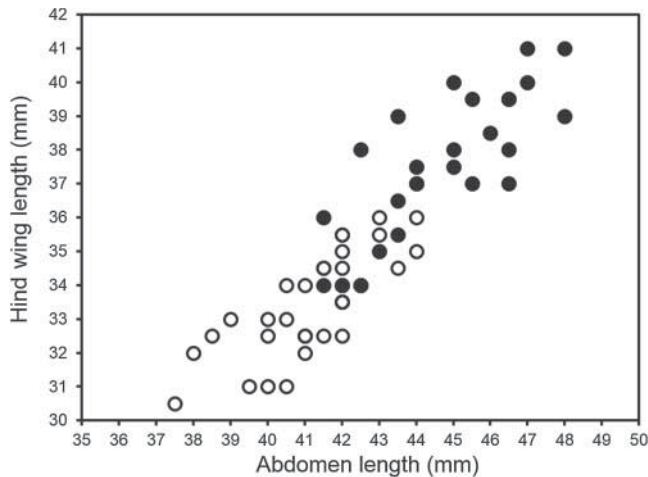


Figure 1. Relationship between abdomen length and left hind wing length in *M. costalis* male (solid circles) and *M. pruinosa* male (open circles).

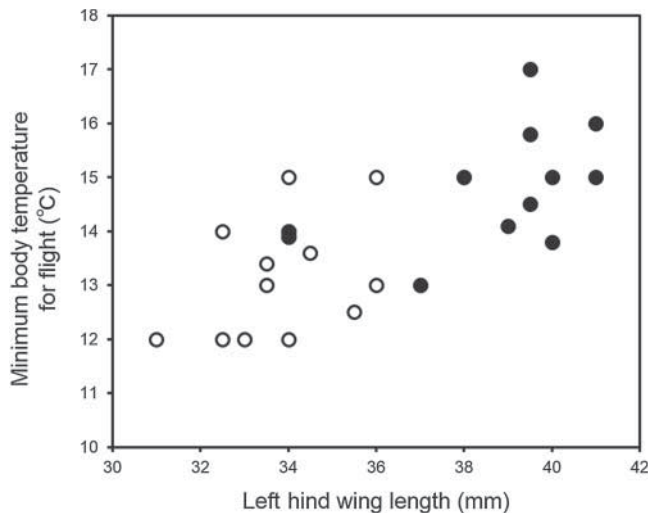


Figure 2. Relationship between minimum body temperature for flight and left hind wing length in *M. costalis* (solid circles) and *M. pruinosa* (open circles).

MBTF

Minimum body temperature for flight (MBTF), that is the thorax temperature at which a damselfly can fly at least horizontally, is shown in Figure 2. Mean MBTFs were 14.76 (SE = 0.322) in *M. costalis*, and 13.24 (SE = 0.285) in *M. pruinosa*. Comparison of MBTF between two species indicated that *M. pruinosa* males could fly at lower body temperature than *M. costalis* males (t value = 3.54, $p < 0.001$) (Figure 2).

Initial thrust production

We measured initial flight speed and wing-beat frequency as measures of initial thrust production. Relations of the initial flight speed to the hind wing length and to the body temperature are shown in Figure 3a, b. The initial flight speed was positively correlated with body temperature

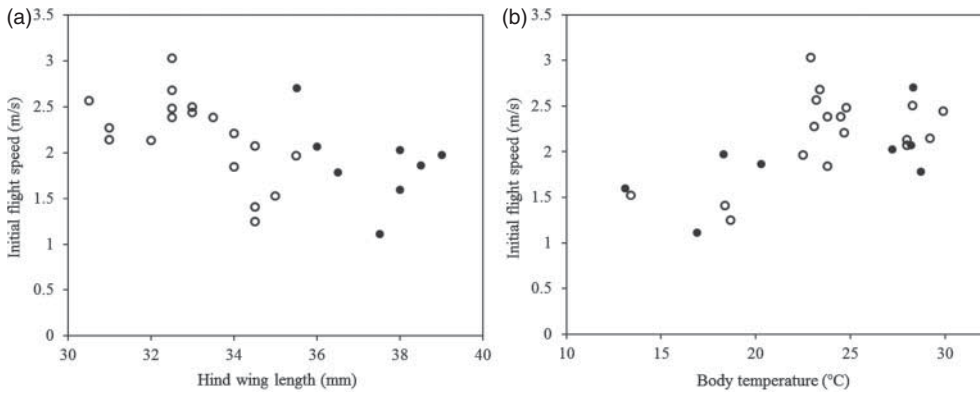


Figure 3. Relationships of the initial flight speed to (a) hind wing length and (b) body temperature in *M. costalis* (solid circles) and *M. pruinosa* (open circles).

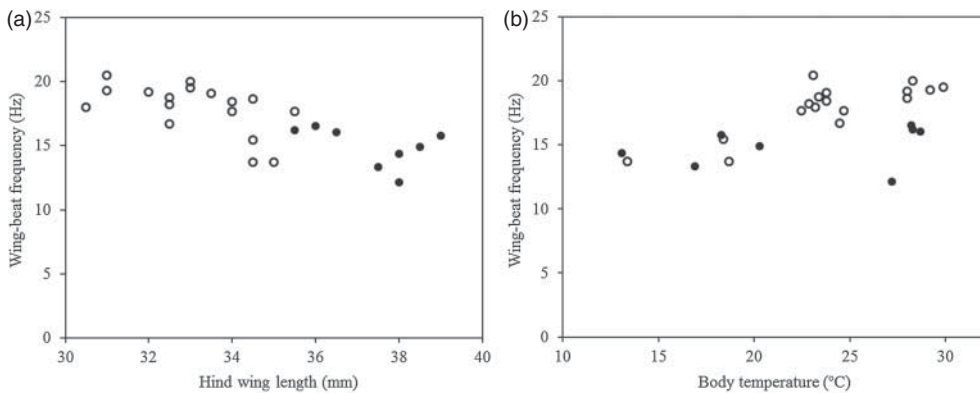


Figure 4. Relationships of the wing-beat frequency to (a) hind wing length and (b) body temperature in *M. costalis* (solid circles) and *M. pruinosa* (open circles).

(estimate = 0.037, $p = 0.0463$) and negatively correlated with body size (estimate = -0.142 , $p = 0.0411$), irrespective of species (estimate = -0.180 , $p = 0.2642$). Relations of the wing beat frequency to the hind wing length and to the body temperature are shown in Figure 4a, b. The wing beat frequency was positively correlated with body temperature (estimate = 0.140, $p = 0.0364$) and negatively with body size (estimate = -0.553 , $p = 0.0275$). There was no significant difference in the initial flight speed between *M. costalis* (mean = 1.89, SE = 0.23) and *M. pruinosa* (mean = 2.22, SE = 0.12) (t -test, $t = 1.652$, $p = 0.1335$), though the wing beat frequency of *M. pruinosa* (mean = 17.79, SE = 0.46) was significantly higher than that of *M. costalis* (mean = 14.92, SE = 0.81) (t -test, $t = 3.578$, $p = 0.0016$).

Discussion

In this study, we showed experimentally that the body size and body temperature influenced two different aspects of flight performance; minimum body temperature for flight (MBTF), and initial thrust production. Regarding MBTF, May (1976) analyzed the relationship between MBTF and body weight of dragonflies belonging to various taxonomic groups. May showed that the large species, e.g. *Anax* and *Macromia*, required MBTF of about 25°C, whereas small species

such as *Miathyria* and *Ladona* required MBTF of about 20°C. In this study, we detected body size dependency and difference in MBTF between closely related sympatric species. On average, MBTFs for *M. costalis* and *M. pruinosa* are about 15°C and 13°C respectively. MBTFs measured on *Mnais* damselflies (Zygoptera) are much lower than the measurements on anisopteran dragonflies (May, 1976). This may be partly because damselflies generally have lower wing-loading than anisopteran dragonflies, and partly because *Mnais* damselflies are basically forest dwellers and adapted to shady environments or to cold climate conditions.

We suggest that the difference in MBTF between *M. costalis* and *M. pruinosa* might be associated with character divergence driven by species interactions between the two species. We have previously demonstrated microhabitat segregation between *Mnais* damselflies (Okuyama et al., 2013), and character divergence through loss of a wing color polymorphism is also linked to microhabitat segregation of two species (Okuyama et al., 2013; Suzuki & Tamaishi, 1982). In *M. costalis*, the loss of polymorphism occurs through the disappearance of clear-winged males, while in *M. pruinosa* the loss of polymorphism occurs through the disappearance of orange-winged males. As the orange-winged morph is larger than the clear-winged male in polymorphic populations of both species (Nomakuchi et al., 1988; Tsubaki et al., 1997), the loss of polymorphism may simply result in divergence in body size. So, the mean body size of monomorphic *M. costalis* populations is expected to be larger than that of polymorphic populations. On the other hand, the mean body size of monomorphic *M. pruinosa* populations is expected to be smaller than that of polymorphic populations. Our present study suggests that microhabitat segregation observed in the two species is associated with body-size dependency of thermoregulation.

In some cases where species coexist, ecological interactions and reproductive interference lead to diversification in relative body sizes (Brown & Wilson, 1956). Most previous studies suggest that the diversification in body size is associated with resource partitioning (see review in Pfenning & Pfenning, 2009). In this study, we suggest that the diversification in body size reflects the difference in thermal condition of the preferred microhabitats. Both sexes of the two *Mnais* species spend early morning and late afternoon feeding at feeding sites (usually tree canopy, as usual for calopterygid damselflies) in sympatric microhabitats. They appear to use the same food resources (small flying insects). Therefore, it is unlikely that body size diversification was driven by foraging competition. We suggest that past interspecific reproductive interference and/or competition for territorial space drove microhabitat segregation, into sunny and shady environments. It is not clear why *M. costalis* chose sunny spaces while *M. pruinosa* chose shady spaces; however the orange wings of *M. pruinosa* might not be an efficient sexual signal to attract females in the shade, while the clear wings of *M. costalis* might not be cryptic enough to practice a sneaker strategy in the sun. To confirm this explanation, however, we need to compare microhabitat preference and reproductive behavior of *Mnais* damselflies between sympatric and allopatric populations in future studies. In support of this explanation, we found that both *M. costalis* and *M. pruinosa* have similar preference for semi-shady forest environments in allopatry and they show microhabitat segregation only when they are sympatric (Tsubaki & Okuyama, 2015).

It is usually accepted that flight speed is correlated with a product of wing length and wing beat frequency in insects (Sane, 2003). We detected influences of body size and body temperature on the initial thrust production; however, species differences in initial flight speed were not detected. This may be partly because of small sample size, but it seems that body size dependency in the wing-beat frequency decreased the difference in the flight speed.

Our recent geographical survey of two *Mnais* damselfly species showed that the mean body size of the orange morph was consistently larger than that of the clear morph in allopatry (Tsubaki & Okuyama, 2015). The mean body size of the sympatric orange morph of *M. costalis* males was even larger than that of allopatric orange morph males. In contrast, the mean body size of the

sympatric clear morph of *M. pruinosa* males was similar to that of allopatric clear morph males (Tsubaki & Okuyama, 2015). These observations together with the present study suggest that the larger body size of males coupled with courtship display using pigmented wings is probably driven by sexual selection. In fact, larger *M. costalis* males in the sun enjoy higher reproductive success than other males (Tsubaki and Samejima, unpublished data). However, territorial males in the sun are subject to higher survival cost (Tsubaki and Samejima, unpublished data) partly because of their higher metabolism rate due to high body temperature (Okuyama et al., 2013; Samejima & Tsubaki, 2010). In contrast, *M. pruinosa* males do not appear to take this risk as they spend their time mostly in the shade without showing any courtship display toward females.

In conclusion, the loss of color polymorphism and diversification in the body size in sympatry may be adaptive character displacement in each species that occurred after the habitat segregation toward sunny versus shady microhabitats. It is usually difficult to evaluate the cost of reproductive interference on wild populations, because most populations have already achieved almost complete reproductive isolation mechanisms through synergistic effects of character displacements in signal traits, habitat preference, and increased ability of species recognition. However, there are several interesting *Mnais* populations, particularly in sympatry/allopatry boundary zones, where these isolation mechanisms appear to be insufficient (Tsubaki, unpublished data). Physiological and behavioral studies comparing sympatric and allopatric populations are especially important and may contribute to our understandings of the role of interspecific interactions in the process of trait evolution, not only for damselflies but also a wide range of animals.

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